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Leukocyte migratory responses to apoptosis

The attraction and the distraction

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An expanding body of evidence demonstrates that cells undergoing apoptosis send out a selection of molecular navigational signals including proteins, lipids and nucleotides that serve to recruit phagocytes to the dying targets, which are subsequently engulfed and removed. This homeostatic process is essentially non-phlogistic, contrasting markedly with the acute inflammatory responses elicited in phagocytes by damaging or infectious agents. The “professional” scavengers of apoptotic cells are mononuclear phagocytes—the macrophages—and sites of high-rate apoptosis are clearly characterized by macrophages associated with the apoptotic cells. By contrast, members of the other class of professional phagocytes—the granulocytes—are not recruited to sites of apoptosis as a direct consequence of the cell-death program. Indeed, recent work indicates that apoptotic cells release a mixture of migratory cues to leukocytes in order to selectively attract mononuclear phagocytes but not granulocytes through functional balancing of positive and negative signals. Here we discuss these molecular mechanisms that not only serve as migratory cues but also may activate responding phagocytes to engulf apoptotic cells effectively. Finally, we speculate upon new therapeutic opportunities these mechanisms offer for a range of pathological conditions, including inflammatory disorders and cancer.

not least of which are the responses of phagocytes that locate, recognize, engulf and degrade dying cells. The efficiency and importance of these phagocytic responses is illustrated by the paucity of readily detectable apoptotic cells in tissues and by the pathological consequences of persistent apoptotic cells, particularly illustrated in animal models in which specific pathways in these responses are blocked.^{1–10} Recently, knowledge of the molecular mechanisms underlying the “scent” of cell death¹¹—the mechanisms by which phagocytes initially locate and navigate towards apoptotic cells—has increased substantially. Predictably, as the molecular complexity of the chemoattractive mechanisms unfolds, additional questions arise, some of which we will address here. Specifically, given that phagocyte chemoattractant moieties released by apoptotic cells have potential to act on both groups of professional phagocytes—both the mononuclear phagocytes and the granulocytes—how is it that only mononuclear phagocytes are actively recruited to locations of apoptosis? Furthermore, since the chemoattractants of phagocytes have multifunctional properties, are these important in the apoptotic-cell clearance process? Finally, can these migratory mechanisms be targeted therapeutically?

Phagocyte Chemoattractant Signals Coupled to Clearance of Dead and Dying Cells

We have recently rationalized three main phases in the clearance of apoptotic cells: recognition, response and removal.¹⁰

Introduction

Inherent in the cell-death program apoptosis are cellular responses to the program,

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Acting early in the recognition phase are the molecules that signal the location of the apoptotic cell to phagocytes. Immediately juxtaposed cells of a variety of different lineages—including for example, fibroblasts, epithelial cells and myocytes—can phagocytose their apoptotic neighbors and although little is yet known of the signaling processes underlying such “non-professional” phagocytic responses, (cf. ELMO1 in clearance of apoptotic germ cells in the testis¹²), it is assumed that the early migratory responses are generally not required. It is conceivable, however, that additional functional (e.g., pro-phagocytic, *vide infra*) effects of the same chemoattractants as those required for recruitment of professional phagocytes may be required for the response phase of non-professional phagocytes of apoptotic cells. We will consider here the responses of the professional phagocytes, since these are invariably found at sites of apoptosis in multiple tissues, embryonic and adult.

Over recent years, the spectrum of chemoattractants produced by apoptotic cells to activate directed migration of mononuclear phagocytes has grown markedly. The best characterized players now include the lipids lysophosphatidylcholine (LPC) and sphingosine-1-phosphate (S1P),^{13,14} the nucleotides, ATP and UTP,^{15,16} as well as the classical protein chemokines, CX₃CL1 (fractalkine) and CCL2 (MCP-1).^{17,18} All of these molecules appear to act through phagocyte-associated G-protein-coupled receptors—G2A (for LPC), P2Y₂ (for nucleotides), CX₃CR1 (for CX₃CL1), CCR2 (for CCL2) and ill-defined GPCR (for S1P).^{13–15,17,18} Other phagocyte chemoattractant proteins released from apoptotic cells include the covalent dimer of ribosomal protein S19, endothelial monocyte-activating polypeptide II (EMAP II), thrombospondin 1, TGFβ and annexin I (reviewed in refs. 9 and 19).

Several important questions arise in connection with the attributes of such a panoply of potential chemoattractants: (1) Which molecules are actively released from stressed (pre-apoptotic) cells, dying (apoptotic) cells or passively released from dead (lysed, necrotic, post-apoptotic cells)? (2) Which ones are specifically chemotactic for (or enable directed migration of) mononuclear phagocytes? (3) Do these

molecules have additional functions of relevance to the clearance process (response and removal phases)? (4) Which molecules predominate in supporting clearance of dying and dead cells in normal or pathological processes *in vivo*?

Phagocyte Chemoattractants Released from Stressed, Apoptotic and Dead Cells

An extensive array of small molecules and macromolecules such as heat shock proteins, nucleotides and formylated peptides that have the potential to act as phagocyte chemoattractants are released from cells whose plasma membranes have lost integrity.^{20–23} Such cells would include those that undergo primary necrosis and those that reach the post-apoptotic state—a situation that occurs in pathological conditions and also occurs frequently *in vitro* when, as is usually the case with cell cultures, apoptotic cells persist because of the absence of effective phagocytic clearance mechanisms. Persistence of apoptotic cells does not occur under normal circumstances *in vivo*, although can occur when molecular clearance pathways are blocked and has been linked to autoimmune disease (reviewed in ref. 8).

Of importance to the biological value of apoptosis and to the phagocytic clearance process is the essence of speed: phagocytes responding at a distance require time to sense and locate apoptotic cells. To this end, cells committed to undergo apoptosis would be expected to produce chemoattractant/pro-migratory signals actively at an early stage in the process, in concert with the molecular machinery of the apoptosis program. Indeed, it seems likely that stressed cells that are not of necessity committed to undergo apoptosis—but rather are pre-apoptotic, with commitment to the cell-death program a fate decision still to be concluded—may also send out earlier navigational signals to phagocytes. Since phagocytes have the ability either to promote cell death or cell survival,^{24–27} such signals (perhaps depending additionally on phagocyte activation properties of migration-controlling factors, as we discuss later) could conceivably either promote or prevent commitment to apoptosis in pre-apoptotic, stressed cells.

In this way, the phagocyte response could be pivotal in certain circumstances to the fundamental fates of cell death or survival.

While several studies associate chemoattractant release with the activation of the apoptosis program and demonstrate active release prior to plasma membrane breakdown (reviewed in refs. 14, 15 and 17), such investigations tend not to differentiate between processes occurring in stressed cells that lead to apoptosis and mechanisms activated specifically in apoptosis. Both situations are likely to have significant biological value. Other studies provide compelling arguments for a mechanistic association of chemoattractant release and the activation of the apoptosis machinery by demonstrating that effector proteases in the apoptosis program act on key substrates that consequently mediate release of chemotactic molecules. Thus, Lauber and colleagues showed that release of the chemotactic lipid LPC from apoptotic cells is dependent on the activation of phospholipase A2 (which hydrolyses membrane-associated phosphatidylcholine to produce LPC) by the apoptotic effector protease, caspase-3.¹³ More recently, Ravichandran’s group demonstrated that the release of nucleotides in apoptosis occurs via the plasma membrane channel pannexin 1 (PANX1). Significantly, PANX1 was found to be a target in apoptosis for the effector proteases caspase-3 and caspase-7 with a specific caspase cleavage site in the channel being required for its regulation during the apoptosis program.¹⁶

Specific and Combinatorial Signals for Selective Attraction of Mononuclear Phagocytes

Regardless of whether chemotactic factor release occurs as a specific consequence of activation of the apoptosis machinery or as a result of pre-apoptotic stress (both are biologically relevant), how is selective attraction of mononuclear phagocytes ultimately achieved? This is an important consideration because it is clear that factors which have the capability to effect attraction of both types of professional phagocytic leukocytes—mononuclear and polymorphonuclear (granulocytes)—are released from apoptotic cells. For example,

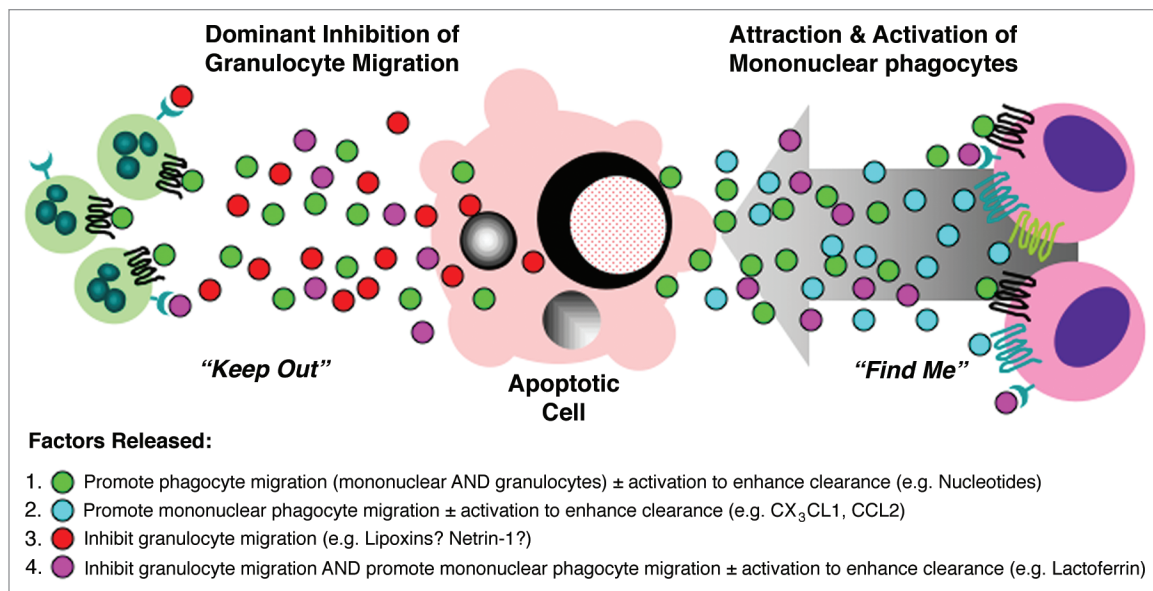


Figure 1. Leukocyte migratory factors released from apoptotic cells: "Keep Out" and "Find Me" signals. Selective attraction of mononuclear phagocytes to sites of apoptosis is proposed to result from a balance between positive signals that attract leukocytes (or specifically mononuclear phagocytes) and negative, anti-inflammatory signals that inhibit granulocyte migration. Four categories of signaling factors are proposed: (1) factors that promote migration of both mononuclear phagocytes and granulocytes (e.g., ATP, EMAP II); (2) factors that promote selective migration of mononuclear phagocytes (e.g., CX₃CL1, CCL2); (3) factors inhibiting granulocyte migration (none yet defined as released from apoptotic cells but candidates include lipoxins and netrin-1); (4) factors that both attract mononuclear phagocytes but inhibit granulocyte migration (e.g., lactoferrin). The promoters of monocyte/macrophage attraction may also prepare these professional phagocytes for enhanced clearance of apoptotic cells. Adapted from reference 11.

ATP is known to act as a chemoattractant (or at least an activator of migration) for both granulocytes and mononuclear phagocytes but granulocytes are not recruited to sites of apoptosis to mediate cell clearance whereas mononuclear phagocytes are. Similar arguments can be made for the cytokine-like polypeptide EMAP II which also has functional activity in promoting granulocyte chemoattraction.^{11,28,29} Recent work suggests a possible mechanism of selective recruitment of professional phagocytes whereby release of multiple regulators of migration by apoptotic cells functionally 'filters out' granulocytes resulting in preferential mononuclear phagocyte attraction (Fig. 1). The paradigm for this mechanism that comprises a balance of positive and negative migratory cues ("Find Me" versus "Keep Out" signals¹¹) lies in the developing nervous system. Thus, we suggest that a key molecule in this filtration process is lactoferrin—a pleiotropic protein with anti-inflammatory properties which we reported to be released from apoptotic cells of multiple cell lineages.³⁰ Even primary human lymphocytes were found to

release lactoferrin in response to apoptotic stimuli. Significantly, we showed that lactoferrin binds to specific receptors on granulocytes and markedly inhibits their ability to migrate towards numerous chemoattractants in vitro or to respond to acute inflammatory stimuli in vivo.^{30,31} By negatively regulating granulocyte activation and migratory responses, apoptotic cells are thus able to deploy a barrage of leukocyte pro-migratory molecules—including those that may otherwise recruit granulocytes—in pursuit of macrophage attraction (Fig. 1).

Our studies indicated that other negative regulators of granulocyte migration are also likely to be produced by apoptotic cells because residual migration inhibitory activity was observed in the absence of lactoferrin or when its function had been neutralized.³⁰ Indeed, although to our knowledge no other specific negative regulators of neutrophil recruitment released from apoptotic cells have been directly implicated, (although annexin 1 may be a candidate in this respect in view of its ability to inhibit neutrophil-endothelial binding under conditions of flow³²) there are

other potential candidate molecules that could fulfill this role. For example, the laminin-related neural guidance protein, Netrin-1 functionally inhibits neutrophil recruitment through attenuation of trans-epithelial migration.^{33,34} Furthermore, depending on the local concentrations and site of generation, agents that elevate cAMP (especially the prostaglandins PGE₂, PGI₂ and PGD₂) in neutrophils may dampen neutrophil recruitment and activation.³⁵⁻³⁸ Other lipids demonstrated, especially by Serhan and colleagues, to play important roles in the resolution of inflammation are the lipoxins, resolvins and protectins.³⁹⁻⁴¹ These lipids generated and released during different phases of the resolving inflammatory response have an intriguing biological profile that are likely important in selective recruitment and activation of specific leukocyte subtypes. Furthermore, it has been well documented that apoptotic cells that interact with macrophages can cause the phagocytes to switch from a pro-inflammatory to a more anti-inflammatory phenotype. Consequently, there is a downregulation of pro-inflammatory mediator generation

and release (e.g., IL-1, IL-8 and TNF α) with pro-active release of mediators with anti-inflammatory potential (including IL-10, TGF β and PGE $_2$).^{42,43} This change in the inflammatory milieu per se may favor differential recruitment of different leukocyte populations. Thus future studies will undoubtedly identify what specific environmental factors and/or other negative regulators of neutrophil recruitment are responsible for the leukocyte profile seen at sites of apoptotic cell clearance, including inflammatory sites and tumors.

Phagocyte-Activating Properties of Chemoattractants

In addition to providing critical migratory cues to phagocytes, chemoattractants released from apoptotic cells also appear to activate mononuclear phagocytes in order to improve their preparedness for apoptotic cell removal (Fig. 1). Presumably by divergent intracellular signaling pathways or differential receptor usage, apoptotic cells thus seem capable of conditioning phagocytes remotely, not only to locate them but also to make provision for effective recognition and removal. For example, interaction of the chemokine CX $_3$ CL1 with its receptor on macrophages activates the phagocytes to produce MFG-E8, a bridging molecule that connects phagocyte α_v integrins with the phosphatidylserine that is exposed on apoptotic cells, thereby promoting engulfment.⁴⁴ In addition, other factors such as annexin I, IL10 and TGF β that are released from apoptotic cells not only have reported chemoattractant activity for mononuclear phagocytes (or in some cases at least have indirect capacity to activate chemotaxis via specific chemoattractant induction) but also have capability in promoting apoptotic-cell clearance.⁹ Furthermore, the ATP that is released from apoptotic cells has potential to enhance their recognition by macrophages since recent work has demonstrated that extracellular ATP promotes, via P2X $_1$ or P2X $_3$ receptors, macrophage binding of apoptotic cells.⁴⁵ Lactoferrin has also been found to function as a chemoattractant for mononuclear phagocytes⁴⁶ and it will be important to determine whether this highly pleiotropic

glycoprotein also activates macrophages to phagocytose their apoptotic targets more effectively, particularly since phagocytic responses to pathogens in macrophages are stimulated by lactoferrin.⁴⁷ Further work will be required to determine the full extent of the molecular mechanisms underlying phagocyte “activation-for-clearance” properties of leukocyte migration regulatory molecules released by apoptotic cells.

Conclusions

The phagocytic cues emanating from apoptotic cells are both complex and curious, and much research is needed in order to expand the currently limited level of understanding of the underlying molecular mechanisms. There is no doubt that the molecules that regulate leukocyte responses to the apoptosis program have broad therapeutic potential. For example, suppression of macrophage recruitment to high-grade tumors (in which apoptosis abounds) through antagonistic targeting of the molecular players is likely to have negative influences on tumor growth, especially in view of the pro-tumor functions of tumor-associated macrophages (TAM). Furthermore, inhibition of effective interaction of TAM with apoptotic cells could conceivably lead to persistence of dead cells, pro-inflammatory sequelae and subsequent anti-tumor responses. As we have previously rationalized,³⁰ antagonism of lactoferrin’s anti-inflammatory effects in the microenvironment of high-grade malignancies could also facilitate anti-tumor immune responses, including those elicited by granulocytes, recruited as a consequence of relaxation of the mechanisms that otherwise inhibit their migration. By contrast, agonists of the leukocyte migratory control mechanisms generated by apoptosis have great potential to be used to treat inflammatory disorders, allergies and wounds. In conclusion, the future holds great promise, not only in improving our understanding of the “outreach” of apoptosis to the innate immune system, but also in applying that knowledge to the development of new therapies to a range of pathological conditions in which apoptosis is a prominent process.

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